Influence of glutethimide on rat brain mononucleotides by sub-chronic codeine treatment

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Received: September 16, 2001; Accepted: October 27, 2001

Abstract

It was investigated the *in vivo* effect of glutethimide on the intracellular neuroadaptation characteristic for μ -opioid receptor tolerance induced by chronic codeine treatment and reflected by increased levels of adenylyl cyclase (AC) and cAMP-dependent protein kinase (PKA). AC activity was appreciated by cyclic-AMP (cAMP) formation, the levels of adenine and guanine nucleotides in brain extracts being assayed using a high performance liquid chromatographic method. The concomitant chronic administration of codeine and glutethimide resulted in a pronounced and long-lasting energetic depletion of the neurons, consistent with the high risk of overdose, and increase of cAMP's stable metabolite, 5'-AMP. This increase is persistent even after withdrawal and suggests an interference with the adenylyl cyclase system involved in the development of tolerance of opioid receptor and in relapse and provides a possible explanation of addiction and fast increase of doses observed in humans abusing this combination.

Keywords: codeine • glutethimide • chronic treatment • cAMP accumulation • tolerance

Introduction

The opioid derivative codeine has low affinity for the opioid receptors and consequently, the analgesic and euphoric effects are reduced, being correlated primary to its conversion to morphine [1]. However, the combination of codeine with the enzyme inducer glutethimide (Doriden) was largely abused in the

United States in the '70-80s of the last century, being known in the drug culture as "loads", "hits" or "sets". The combination produced a state of euphoria comparable to heroin and lasting 6 to 8 hours [2-5]. Abusers become quickly addicted and experienced increased tolerance with high risk of overdose, "hits" deaths occurring more frequently and being younger than heroin deaths [2].

Recent studies on molecular mechanisms of drug reinforcement and addiction provided evidence that certain intracellular proteins associated with adenosine-3', 5'-cyclic-monophosphate (cAMP)

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second messenger and protein phosphorylation system may mediate acute drug reinforcement and show adaptive up-regulation following chronic drug exposure [6]. Opioid receptors belong to the family of membrane-bound receptors coupled with G proteins and using cAMP as one of their second messengers. Stimulation of these receptors inhibits adenylyl cyclase (AC) activity, decreasing in a dose-dependent manner the synthesis of cAMP. Opioid antagonists such as naloxone antagonize these effects. Experiments with NG 108-15 neuroblastoma-glioma cell cultures have shown that opioid-induced inhibition of adenylyl cyclase is attenuated when cells are treated chronically with opioid agonists, and the addition of naloxone to cells treated chronically with morphine produces an increase in adenylyl cyclase (AC) above control levels. On the basis of these effects, some researchers have suggested that this system might provide a model of tolerance and dependence [7]. Although pharmacokinetic changes may mediate some aspects of opioid-induced tolerance, mechanisms such as receptor down-regulation, desensitization and alterations in opioid receptor signal transduction are thought to play a major role in opioid tolerance [6]. A possible pathway explaining μ -opioid receptor tolerance is that of the cAMP dependent protein kinase (PKA), both AC and PKA activities being increased as tolerance develops [8]. This mechanism was proposed for G-protein coupled receptors coupled with G_s , which positively regulates the adenylyl cyclase and cAMP levels, and may be acting as a negative feedback mechanism. However, other studies have revealed that PKA is not involved in the development of μ -opioid receptor tolerance to morphine, although the phosphorylation of this receptor is important for the development of tolerance [9]. It was proven that chronic exposure to opiates results in neuroadaptations in the Nucleus accumbens (NAc) cAMP pathway, that are characterized by decreased levels of adenylyl cyclase and PKA activity, that can persist for several weeks after withdrawal. These neuroadaptations may represent an intracellular mechanism of tolerance and rewarding properties of drugs [10]. Modulation of the cAMP pathway in the NAc has been related directly to relapse and drug-seeking behavior and, due to the long-lasting risk of relapse, it is possible that the neuroadaptations are mediated via drug-induced changes in gene

expression. Thus, chronic morphine has been shown to decrease the level of transcription factor cyclic AMP response element-binding protein (CREB) in NAc [11]. The changes in the cAMP system in response to chronic morphine administration are not specific only to opioids, but also to other drugs such as Δ^9 -THC (delta-9-tetrahydrocannabinol), where the increase in the cAMP system was observed throughout all the brain regions expressing CB₁ (cannabinoid-1) receptors [12].

While in United States and Western Europe glutethimide has been replaced with other sedative-hypnotics, in Eastern Europe this drug as well as codeine is still easily available and the use of this or similar combinations is constantly growing.

The aim of this study was to verify if the chronic administration of codeine-glutethimide association induces persistent neuro-adaptations that can explain the increased tolerance and addiction experienced by the abusers.

Materials and methods

Animals

In the experiment were included 3 experimental groups and a negative control group, each consisting of 40 male Wistar rats (body weight 120 ± 10 g). The animals were supplied by the Animal Breeding Facility of the University of Medicine and Pharmacy Cluj-Napoca and were maintained in standard food and microclimate conditions during the whole period of observation, the studies compiling with legislation governing experimentation on animals.

Reagents

Reagent-grade nucleotides (GTP, GDP, ATP, ADP, 5'-AMP, 3'-AMP, 3', 5'-cAMP, 3', 5'-cGMP) and $NH_4H_2PO_4$ were purchased from Sigma, and methanol of HPLC purity from Merck. Codeine phosphate and glutethimide were of pharmaceutical purity and were obtained from Terapia S.A., Cluj Napoca.

Treatment

The substances were administered for 90 days, orally by nasal intubation, codeine as phosphate salt dissolved in distilled water and glutethimide in aqueous suspension prepared with Tween 80.

The doses were:

- Experimental group I: a daily dose of 1/10 LD₅₀ of codeine phosphate (42 mg/kg), associated with 1/10 LD₅₀ of glutethimide (60 mg/kg);
- Experimental group II: a daily dose of codeine phosphate equivalent to 1/10 of LD₅₀ (42 mg/kg);
- Experimental group III: a daily dose of glutethimide equivalent to 1/10 LD₅₀ (60 mg/kg).

The negative control (Group IV) received the same amount of distilled water with Tween 80 as group I.

Sampling

On days 30, 60, 90 of the experiment and 30 days after the cessation of the administration, 10 rats from each group were anesthetized with diethyl ether, sacrificed by decapitation and the whole brain was taken immediately on ice for nucleotide assay.

Nucleotide extraction

Nucleotide extraction from the whole brain following homogenization was performed according to Perret method [13] by protein precipitation with trichloroacetic acid 10% (1 ml for 100 mg tissue), centrifugation 10 min. at 2000g in a cooling centrifuge and neutralization of the supernatant with potassium hydroxide 1M. The lipids were removed by extraction with diethyl ether.

Nucleotide separation

Nucleotide separation was performed by a reversed phase HPLC method, using an ODS column (200 x 4 mm i.d. and 5 µm particle diameter) and a gradient system consisting of

two Altex 101 pumps and a UV-VIS Kontron Instruments detector coupled to a Hewlett Packard 3650 integrator [14]. The chromatograms were registered at 254 nm and the flow rate of the mobile phase was 1.5 ml/min. The elution of the samples was performed with a step-gradient of 0.05 M NH₄H₂PO₄ (solvent A, pH = 6) (0-5 minutes) and a mixture 90:10 of 0.05 M NH₄H₂PO₄: methanol (solvent B, pH = 5.5, from 5 to 25 minutes).

Statistical analysis

The results as percentage from controls were presented as mean values ± standard deviation, and the values for the experimental group I (association) were compared with the experimental groups II (codeine) and III (glutethimide) by ANOVA analysis of variance and post-tests using INSTAT computer software.

Results

The identification of the nucleotides in the biological samples was done according to their retention times (*t_R*) and the order of elution compared with standards' separation (Fig. 1). To confirm the identification of the peaks, co-chromatograms of samples with different standard nucleotides (as mentioned in Materials and Methods) were also performed. For the quantitative analysis, the chromatographic peaks were integrated and from their area, the percentage of each nucleotide was computed.

Fig. 2a shows the separation of adenine and guanine nucleotides from brain extracts of control

Fig. 1 HPLC separation of nucleotide standards GTP + GDP (*t_R* = 1.64 min.), ATP (*t_R* = 2.27 min.), ADP (*t_R* = 2.85 min.), 5'AMP (*t_R* = 6.30 min.), 3'AMP (*t_R* = 13.13 min.), cGMP (*t_R* = 14.58 min.) and cAMP (*t_R* = 20.93 min.). For details see Materials and methods. *t_R* = retention times.

Fig. 2 HPLC separation of brain adenine and guanine nucleotides (a): control rat, day 90 of the experiment; (b): determination at day 30, after the 90 days administration of the association codeine-glutethimide to rats.

rats, the following nucleotides being identified: GTP co-eluted with GDP, ATP, ADP and 5'-AMP. The signals with $t_R = 14.58$ min., corresponding to 3', 5'-cGMP and with $t_R = 20.93$ min., corresponding to 3', 5'-cAMP in the standard mixture (Fig. 1) were not present in the biological samples. As only the basal, non-stimulated AC activity was investigated and 3', 5'-cAMP (whose levels are normally below 10^{-6} M) is metabolized within seconds to 5'-AMP [15, 16], the effects of the tested substances on this second messenger were appreciated by the variation of 5'-AMP levels, the stable metabolite of cAMP. Fig. 2b shows the separation of adenine and guanine nucleotides in a brain extract from a rat treated for 90 days with codeine-glutethimide association and sacrificed 30 days after the cessation of the administration. In the chromatogram were identified the same peaks but with different areas, a decrease in the percentage of macroergic compounds (ATP) in the neuronal cell and a relative increase in the percentage of 5'-AMP peak compared to the other components being observed.

Fig. 3 presents the evolution compared to control of adenine and guanine nucleotides percentage at brain level during the sub-chronic

administration of codeine and glutethimide as such and in association. In all experimental groups, a decrease in the ATP percentage in the neuronal cell was observed. The decrease was more pronounced in codeine group following 30 days, and was observed after 90 days in all groups, having statistical significance in the association group ($p < 0.001$ compared to glutethimide group), where the decreasing tendency was maintained even 30 days after the cessation of the administration ($p < 0.001$ compared to codeine and to glutethimide groups). It was also observed that the GTP and GDP percentage increased during the first 60 days of administration in all experimental groups and especially in the association group ($p < 0.001$ compared to codeine and to glutethimide groups), decreased to values comparable to controls following 90 days of codeine administration, but was maintained significantly increased in the association group ($p < 0.001$ compared to codeine and to glutethimide groups) even 30 days after the cessation of the treatment (Fig. 4).

The 5'-AMP percentage was slightly increased in the association group after 30 days of treatment, but the interpretation of the results is difficult as the

Fig. 3 Evolution, compared to control, of adenine and guanine nucleotide from brain extracts, after 30 days (A) and 60 days (B) exposure to codeine (c), glutethimide (g) and codeine-glutethimide association. [***=p<0.001, **=p<0.01, *=p<0.05, ns=p>0.05, (c)=compared to codeine, (g)=compared to glutethimide].

Fig. 3 cont'd Evolution, compared to control, of adenine and guanine nucleotide in brain at 90 days (C), after administration of codeine, glutethimide and codeine-glutethimide association and at 30 days of abstinence (D). [***=p<0.001, **=p<0.01, *=p<0.05, ns=p>0.05, (c)=compared to codeine, (g)=compared to glutethimide].

Fig. 4 Evolution of GTP and GDP (expressed as percentages) during the experiment.

Fig. 5 Evolution of 5'AMP (expressed as percentages) during the experiment.

values are very small in all groups. After 90 days the percentage was increased in all experimental groups and was maintained increased in the association group even after 30 days of abstinence ($p < 0.001$ compared to codeine and glutethimide groups) (Fig. 5). The absence of 5'-AMP in chromatograms of brain extracts from rats sacrificed after 60 days of codeine-glutethimide administration can be explained probably by an experimental error, rather than the effect of the substances. The variation of nucleotide percentages during the experiment is due to inter-individual variability and inherent age differences of the animals.

Discussion and conclusions

Our experiments have reported an energetic depletion of the neuronal cell by a sub-chronic administration of the CNS-depressant drugs as codeine and/or glutethimide, more pronounced and persistent when they were administered concomitantly, consistent with the high risk of overdose and the lethal comatose state observed in human abusers [2]. The increase in the GTP and GDP percentage during the whole period of experiment in the association group could indicate an activation of G_s proteins coupled with μ -opioid receptor that up-regulates adenylyl cyclase and cAMP levels, being involved in the development of tolerance [9]. It was also observed a significant increase in 5'-AMP level in the codeine-glutethimide group following 90 days of exposure, this tendency being maintained even after 30 days of abstinence. Because 5'-AMP is the main and stable metabolite of cAMP, we can suppose that this increase might reflect the development of some neuro-adaptations characteristic for tolerance and which persist a long period after withdrawal, being correlated with the risk of relapse and drug seeking behavior.

These results confirm that the chronic administration of codeine in association with glutethimide determine biochemical changes at the neuronal level and suggest their interference with the adenylyl cyclase system involved in the development of tolerance of opioid receptor and in relapse. These changes are persistent and much more pronounced than those induced by codeine alone and could explain the addiction and the fast increase of doses observed in persons abusing this combination.

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